British Journal of Pharmacology (1998) 123, 1173–1181

Pharmacological comparison of UTP- and thapsigargin-induced arachidonic acid release in mouse RAW 264.7 macrophages

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1 Although stimulation of mouse RAW 264.7 macrophages by UTP elicits a rapid increase in intracellular free Ca^{2+} ($[Ca^{2+}]_i$), phosphoinositide (PI) turnover, and arachidonic acid (AA) release, the causal relationship between these signalling pathways is still unclear. In the present study, we investigated the involvement of phosphoinositide-dependent phospholipase C (PI-PLC) activation, Ca^{2+} increase and protein kinase activation in UTP-induced AA release. The effects of stimulating RAW 264.7 cells with thapsigargin, which cannot activate the inositol phosphate (IP) cascade, but results in the release of sequestered Ca^{2+} and an influx of extracellular Ca^{2+}, was compared with the effects of UTP stimulation to elucidate the multiple regulatory pathways for $cPLA_2$ activation.

2 In RAW 264.7 cells UTP (100 μM) and thapsigargin (1 μM) caused 2 and 1.2 fold increases, respectively, in [^{3}H]-AA release. The release of [^{3}H]-AA following treatment with UTP and thapsigargin were non-additive, totally abolished in the Ca^{2+}-free buffer, BAPTA (30 μM)-containing buffer or in the presence of the $cPLA_2$ inhibitor MAFP (50 μM), and inhibited by pretreatment of cells with pertussis toxin (100 ng ml^{-1}) or 4-bromophenacyl bromide (100 μM). By contrast, aristolochic acid (an inhibitor of $sPLA_2$) had no effect on UTP and thapsigargin responses.

3 U73122 (10 μM) and neomycin (3 mM), inhibitors of PI-PLC, inhibited UTP-induced IP formation (88% and 83% inhibition, respectively) and AA release (76% and 58%, respectively), accompanied by a decrease in the $[Ca^{2+}]_i$ rise.

4 Wortmannin attenuated the IP response of UTP in a concentration-dependent manner (over the range 10 nM – 3 μM), and reduced the UTP-induced AA release in parallel. RHC 80267 (30 μM), a specific diacylglycerol lipase inhibitor, had no effect on UTP-induced AA release.

5 Short-term treatment with PMA (1 μM) inhibited the UTP-stimulated accumulation of IP and increase in $[Ca^{2+}]_i$, but had no effect on the release of AA. In contrast, the AA release caused by thapsigargin was increased by PMA.

6 The role of PKC in UTP- and thapsigargin-mediated AA release was shown by the blockade of these effects by staurosporine (1 μM), Ro 31-8220 (10 μM), Go 6976 (1 μM) and the down-regulation of PKC.

7 Following treatment of cells with SK&F 96365 (30 μM), thapsigargin-, but not UTP-, induced Ca^{2+} influx, and the accompanying AA release, were down-regulated.

8 Neither PD 98059 (100 μM), MEK a inhibitor, nor genistein (100 μM), a tyrosine kinase inhibitor, had any effect on the AA responses induced by UTP and thapsigargin.

9 We conclude that UTP-induced $cPLA_2$ activity depends on the activation of PI-PLC and the sustained elevation of intracellular Ca^{2+}, which is essential for the activation of $cPLA_2$ by UTP and thapsigargin. The $[Ca^{2+}]_i$-dependent AA release that follows treatment with both stimuli was potentiated by the activity of protein kinase C (PKC). A pertussis toxin-sensitive pathway downstream of the increase in $[Ca^{2+}]_i$ was also shown to be involved in AA release.

Keywords: Cytosolic phospholipase A_2; $[Ca^{2+}]_i$; protein kinase C; phospholipase C; uridine 5′-triphosphate (UTP); thapsigargin; RAW 264.7 macrophages

Introduction

Macrophages play a key role in many aspects of acute and chronic inflammation. Stimulation of macrophages by inflammatory stimuli leads to the generation of the arachidonic acid (AA) and eicosanoids. Cytosolic phospholipase A_2 ($cPLA_2$) is the rate-limiting enzyme in eicosanoid biosynthesis (Mayer & Marshall, 1993). Although $cPLA_2$ activities have been demonstrated in many cells, including macrophages, the signalling pathways by which $cPLA_2$ is activated or regulated remain unclear.

To date, two biochemical events have been identified as key regulatory factors in the release of AA: an increase in Ca^{2+} availability and protein phosphorylation of $cPLA_2$. It is known that $cPLA_2$ has a Ca^{2+}-dependent phospholipid-binding domain in the amino-terminal region and, in the presence of submicromolar concentrations of Ca^{2+}, it translocates from the cytosol to the nuclear envelope, where it has access to arachidonyl-containing phospholipid substrates (Clark *et al*., 1991; Peters-Golden & McNish, 1993; Schievella *et al*., 1995; Kan *et al*., 1996). An increase in cytosolic Ca^{2+} levels has been proposed as the primary event in regulating AA release by acting directly on $cPLA_2$ and promoting its association with the membrane, thus facilitating AA hydrolysis (Clark *et al*., 1991). It has been shown that $cPLA_2$ is activated by phosphorylation via various protein kinases. $cPLA_2$ activity is regulated by the activation of p42 mitogen-activated protein kinase (MAPK), which can be either protein kinase C (PKC)-dependent or -independent, depending on the stimulus and cell type in question (Wijkander & Sundler, 1991; Lin *et al*., 1993; Nemenoff *et al*., 1993; Qui & Leslie, 1994; Xing & Insel, 1996).

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With respect to the PKC-independent pathway, two possible cascades might be involved. Firstly, MAPK activation involves $\beta\gamma$ subunits of heterotrimeric G proteins (Crespo *et al*., 1994). Secondly, in rabbit vascular smooth muscle cells, noradrenaline-induced release of AA has been shown to be due to the stimulation of $cPLA_2$ by MAPK via the calmodulin-dependent activation of kinase II (Muthalif *et al*., 1996). p42 MAPK phosphorylation-mediated activation appears to act synergistically with Ca^{2+} to achieve maximal activation (Lin *et al*., 1992; Xing & Mattera, 1992; Tence *et al*., 1994). However, recent studies also suggest that phosphorylation of $cPLA_2$ at multiple sites by protein kinases other than MAPK may be important for the regulation of AA release (de Carvalho *et al*., 1996), such as that observed in human platelets in response to the thrombin agonist (Kramer *et al*., 1996) and in human neutrophils in response to TNFα (Waterman & Sha'afi, 1995).

$cPLA_2$ has been identified in the murine macrophage cell line RAW 264.7, (Channon & Leslie, 1990) and we have demonstrated previously that uridine 5′-triphosphate (UTP) and uridine 5′-diphosphate (UDP) can stimulate phosphoinositide (IP) formation together with AA release, possibly via pyrimidinoceptors (Lin & Lee, 1996). RAW264.7 cells thus provide a unique and attractive model to explore the relevant signalling mechanisms leading to $cPLA_2$ activation by pyrimidinoceptors, which have not been studied previously. Thus, in this study, we investigated the roles of IP formation, intracellular Ca^{2+}, protein kinases and G protein in UTP-stimulated AA release. The results were compared with thapsigargin, which inhibits the Ca^{2+}-ATPase that is essential for the accumulation of Ca^{2+} by endoplasmic reticulum, and can lead to the elevation of $[Ca^{2+}]_i$ without the involvement of inositol 1,4,5-triphosphate (IP_3) formation. Our results provide evidence that UTP-induced AA release is dependent on the activation of phosphoinositide-dependent phospholipase C (PI-PLC), and that the levels of sustained $[Ca^{2+}]_i$ increase primarily determine the degree of $cPLA_2$ activation. PKC activation also potentiated the Ca^{2+}-mediated activation of $cPLA_2$.

Methods

Cell culture

RAW 264.7 cells, generously provided by Dr Yen-Jen Sung (Department of Anatomy, National Yang-Ming Univ. School of Medicine), were grown in 35 mm Petri dishes at 37°C in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% foetal bovine serum, 100 u ml^{-1} penicillin and 100 μg ml^{-1} streptomycin, in a humidified atmosphere of 95% air and 5% CO_2.

[^{3}H]-AA release from intact cells

Cells in 24-well plates (approximately 1.5×10^6 cells/well) were incubated in 5% CO_2 for 24 h with 0.3 μCi ml^{-1} [^{3}H]-AA in DMEM with 10% foetal bovine serum. Cells were then washed three times with physiological saline solution (PSS in mM: NaCl 118, KCl 4.7, $CaCl_2$ 1.8, KH_2PO_4 1.2, glucose 11 and HEPES 20, pH 7.4) and incubated in PSS containing 0.5% fatty acid-free bovine serum albumin and inhibitors as stated for 20 min. Cells were then stimulated with UTP at 37°C for 30 min, after which time the medium was removed and centrifuged at 250 *g* for 5 min to remove floating cells. The radioactivity in the supernatant was then measured.

[^{3}H]-AA release from permeabilized cells

Cells were prelabelled with [^{3}H]-AA, washed twice with PBS and then permeabilized for 5 min at 37°C with 10 μM digitonin in intracellular potassium glutamate buffer (composition in mM potassium glutamate 139, piperazine-N,N′-bis(2-ethanesulphonic acid) 20, $MgCl_2$ 1, MgATP 2 and EGTA 5 (pH 7.4)). The dishes were then washed with potassium glutamate buffer without digitonin containing various concentrations of $CaCl_2$ and then incubated for 30 min. After incubation, the medium was removed and centrifuged at 250 *g* for 5 min to remove floating cells. The radioactivity in the supernatant was then measured. The free $[Ca^{2+}]$ was calculated by use of a computer programme (Fabiato & Fabiato, 1979) to determine the $[Ca^{2+}]$ in the presence of 5 mM EGTA.

Measurement of $[Ca^{2+}]_i$

Cells grown on glass slides were incubated with fura-II/AM (3 μM) and pluronic F-127 (0.02% v/v) in DMEM at 37°C for 45 min. Fluorescence was monitored on a PTI M-series spectrofluorometer with dual excitation wavelengths of 340 nm and 380 nm and an emission wavelength of 510 nm. $[Ca^{2+}]_i$ was calculated as described by Grynkiewicz *et al*. (1985).

Measurement of PI turnover

The hydrolysis of PI was measured in terms of the accumulation of IP in the presence of 10 mM LiCl as described previously (Lin & Lee, 1996). Confluent cells in 35-mm Petri dishes (approximately 8×10^6 cells/dish) were labelled with [^{3}H]-*myo*-inositol (2.5 μCi/dish) in growth medium for 24 h. Cells were then washed twice with PSS containing 10 mM LiCl and incubated at 37°C for 20 min. After this preincubation, the indicated drugs were added and the cells were incubated for another 30 min. The reaction was terminated by aspiration of the reaction solution and addition of ice-cold methanol. The cells were scraped off the dishes and [^{3}H]-IP was isolated with an AG-1X8 column and eluted with 0.2 N ammonium formate/0.1 N formic acid.

Materials

Cell culture medium and its supplements were purchased from Gibco BRL (Grand Island, NY). [^{3}H]-AA (100 Ci $mmol^{-1}$) and [^{3}H]-*myo*-inositol (20 Ci $mmol^{-1}$) were purchased from New England Nuclear (Boston, MA). UTP, fura-II/AM, fatty-acid free bovine serum albumin, straurosporine, thapsigargin, pertussis toxin (PTX), phorbol 12-myristate 13-acetate (PMA), neomycin, wortmannin and 4-bromophenacyl bromide (BPB) were products of Sigma Chemical Co. (St. Louis, MO). 2-(2-amino-3-methoxyphenyl)-4H-1-benzopyran-4-one (PD 98059) and genistein were from RBI (Natick, MA). U73122 (1-[6-[17β-3-methoxyestra-1,3,5-(10) triene-17-yl]amino/hexyl) 1H-pyrroledione), SK&F 96365 (1{β-[3-(4-methoxyphenyl)-propoxy]-4-methoxyphenyl-ethyl}-1H-imidazole hydrochloride) and RHC 80267 (1,6-bis[[[cyclohexylideneamino) oxy] carbonyl]amino]-hexane) were obtained from Biomol (Plymouth Meeting, PA). Ro 31-8220 ([1-[3-(amidinothio)propyl-1H-indoyl-3-yl]-3-(1-methyl-1H-indoyl-3-yl)-maleimide-methane sulphate, 12-(2-cyanoethyl)-6,7,12,13-tetrahydro-13-methyl-5-oxo-indolo(2,3-a) pyrrolo(3,4-c) carbazole (Go 6976) and BAPTA/AM were purchased from Calbiochem (La Jolla,

CA). MAFP was from Cayman (Ann Arbor, MI). AG-1X8 resin (formate form, 100–200 mesh) was obtained from Bio-Rad (Richmond, CA).

Statistical analysis

Each experiment was performed in duplicate and reproduced several times. Data are mean ± s.e.mean values. The significance of the differences between the means was evaluated by Student's t test and a value of $P<0.05$ was considered significant. The error bar was omitted when it was within the symbol representing the mean value.

Results

Calcium influx is necessary for UTP- and thapsigargin-induced AA release

We have previously shown that UTP can increase AA release by RAW 264.7 macrophages in a concentration-dependent manner with an EC_{50} value of 3 μM (Lin & Lee, 1996). UTP 100 μM caused a two fold increase in AA release within 30 min, from a basal level of 740 ± 85 c.p.m. to 2240 ± 148 c.p.m. ($n=33$). Thapsigargin (1 μM) also increased AA release 1.2. fold to 1628 ± 153 c.p.m. ($n=8$). The effects of these two stimuli were found to be non-additive (data not shown).

To investigate the role of Ca^{2+} signalling in the AA response, AA release was obtained for cells grown in a nominally Ca^{2+}-free medium. Following depletion of extracellular Ca^{2+} (removal of $CaCl_2$ and addition of 1 mM EDTA), AA release stimulated by UTP and thapsigargin was completely inhibited and basal AA release was inhibited by 39 ± 4% (Figure 1). Preincubation of cells with 30 μM BAPTA/AM, which leads to the intracellular accumulation of the Ca^{2+} chelator BAPTA, also abolished release of AA in response to both stimuli (Figure 1). SK&F 96365, an inhibitor of capacitative Ca^{2+} entry, was found to inhibit thapsigargin-induced AA release by 70 ± 2% ($n=3$), but had no effect on stimulation by 30 μM UTP (Figure 1).

As shown in Figure 1, BPB (at 100 μM), a non-selective inhibitor of PLA_2, inhibited UTP- and thapsigargin-induced AA release by 61 ± 12% ($n=4$) and 64 ± 2% ($n=3$), respectively. MAFP (50 μM), an inhibitor of cPLA$_2$, abolished both the UTP- and thapsigargin-induced responses. Under the conditions used, neither of the PLA_2 inhibitors alone had a cytotoxic effect, as determined by MTT assays (data not shown). On the other hand, AA release in response to UTP was not affected by aristolochic acid (50 μM), a secretory PLA_2 inhibitor (Vishwanath *et al*., 1988). Pretreatment of cells with 100 ng ml^{-1} pertussis toxin (PTX) for 24 h inhibited AA release in response to UTP and thapsigargin by 58 ± 10% ($n=3$) and 36 ± 1% ($n=3$), respectively.

Activation of PKC has an essential role

In order to elucidate the role of PKC in cPLA$_2$ activation, the effects of PKC inhibitors and activators were tested. As shown in Figure 2, both UTP- and thapsigargin-induced AA release were inhibited by 20 min pretreatment with the PKC inhibitors, staurosporine, Ro 31-8220 and Go 6976. Staurosporine at 1 μM attenuated the response to UTP and thapsigargin by 50 ± 12% ($n=5$) and 33 ± 9% ($n=3$), respectively: Ro 31-8220 at 10 μM reduced these responses by 66 ± 3% ($n=3$) and 53 ± 5% ($n=3$), respectively; and Go 6976 at 1 μM caused reductions in AA release of 87 ± 5% ($n=4$) and 95 ± 3% ($n=3$), respectively. Moreover, while basal AA release was unchanged by staurosporine (1 μM) and Go 6976 (1 μM), it was inhibited by 30 ± 7% ($n=3$) following treatment with 10 μM Ro 31-8220. In contrast, genistein (100 μM), an inhibitor of tyrosine kinases acting on the ATP-binding site (Akiyama *et al*., 1987), and PD 98059 (100 μM), an inhibitor of

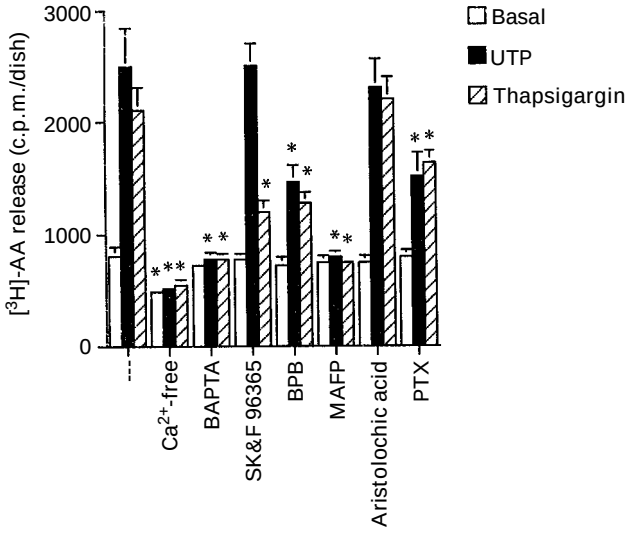

Figure 1 Effects of Ca^{2+}-free media, BAPTA/AM, SK&F 96365, 4-bromophenacyl bromide, MAFP, aristolochic acid and pertussis toxin (PTX) on stimulus-induced and basal AA release. Cells labelled with [^{3}H]-AA were pretreated with Ca^{2+}-free medium, 30 μM BAPTA/AM, 30 μM SK&F 96365, 100 μM 4-bromophenacyl bromide (BPB), 50 μM MAFP or 50 μM aristolochic acid for 20 min, or 100 ng ml^{-1} pertussis toxin (PTX) for 24 h before the stimulation with media only, UTP (100 μM) or thapsigargin (1 μM) for 30 min. The AA released into the medium was collected and radioactivity was counted. Results are expressed as the mean ± s.e.mean of three independent experiments. *Indicates $P<0.05$ as compared to the respective control (basal, UTP or thapsigargin) response without drug pretreatment.

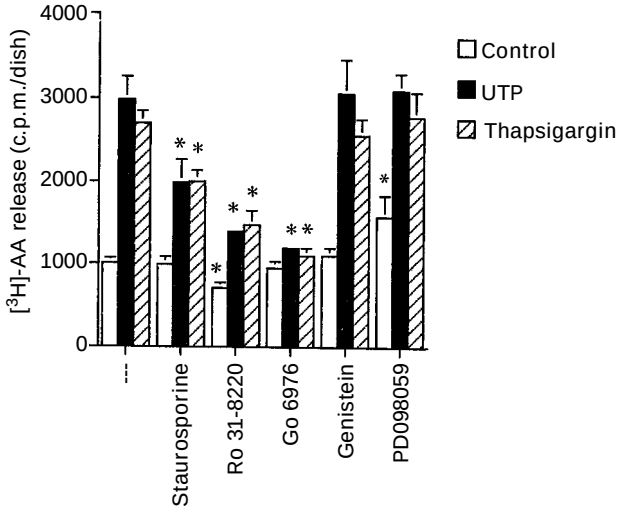

Figure 2 Effects of protein kinase inhibitors on stimulus-induced AA release. Cells were pretreated for 20 min with 1 μM staurosporine, 10 μM Ro 31-8220, 1 μM Go 6976, 100 μM genistein or 100 μM PD 98059 before the stimulation with media only, UTP (100 μM) or thapsigargin (1 μM) for 30 min. Results are expressed as the mean ± s.e.mean of at least three independent experiments. *Indicates $P<0.05$ as compared to the respective control (basal, UTP or thapsigargin) response without drug pretreatment.

MEK (Dudley *et al*., 1995) did not affect AA release in response to either UTP or thapsigargin. Unexpectedly, 100 μM PD 98059 itself caused a slight increase in basal AA release and this action was unrelated to cytotoxicity, as determined from the MTT assay.

Short-term (20 min) treatment of RAW 264.7 cells with 1 μM PMA slightly increased the basal AA release by about 53$\pm$7% (n=12), but did not affect UTP-stimulated AA release. On the contrary, it potentiated the release of AA in response to thapsigargin to about 2 fold (Figure 3a). The concentration-dependent potentiation of thapsigargin stimulation by PMA is shown in Figure 3b. Significant potentiation was seen at PMA concentrations up to 0.3 μM. Whereas in cells pretreated for 24 h with PMA (1 μM), the UTP- and thapsigargin-induced AA responses were inhibited by 54$\pm$7% (n=5) and 57$\pm$3% (n=3), respectively (Figure 3a).

Activation of the PI-PLC systems in UTP-stimulated AA release

To investigate the role of the PI-PLC system in UTP-evoked AA release, the effects of agents that interfere with PI-PLC signalling cascades were tested. In RAW 264.7 cells, UTP induced a significant turnover of PI over the same concentration range at which AA release was seen (Lin & Lee, 1996). Thapsigargin, on the other hand, did not cause an increase in IP formation at concentrations up to 1 μM (data not shown). As shown in Figure 4, pretreatment of cells with U73122 (10 μM), an inhibitor of PI-PLC, reduced UTP-induced IP formation and AA release in parallel by 88$\pm$2% and 76$\pm$4%, respectively. In contrast, U73343 (10 μM), the inactive analogue of U73122, slightly reduced UTP-induced IP formation by 25$\pm$7% (n=3), but did not affect the AA response. Neomycin (3 mM), another inhibitor of PI-PLC, also reduced IP and AA production in response to UTP by 83$\pm$2% and 58$\pm$9%, respectively.

The release of AA is known to occur via two pathways: either due to liberation of AA from phospholipids by PLA$_2$ or to the combined action of PLC (generation of diacylglycerol, DAG) and DAG lipase (liberation of AA from DAG) (Sprang, 1990; Dieter & Fitzke, 1993). The DAG lipase inhibitor RHC 80267 (Balsinde *et al*., 1991; Dieter & Fitzke, 1993) was used to investigate the contribution of the latter pathway to AA release. As shown in Figure 4, RHC 80267 at a concentration previously shown to inhibit DAG lipase (30 μM) had no effect on PI or AA production in response to UTP. In addition to PLC cascades, endogenous phosphatidic acid (PA), released by PLD, and exogenous PA have been shown to potentiate the activation of PLA$_2$ in cooperation with Ca^{2+} in rabbit platelets (Hashizume *et al*., 1994). To explore this possibility, we tested propranolol (an inhibitor of PA phosphohydrolase) and

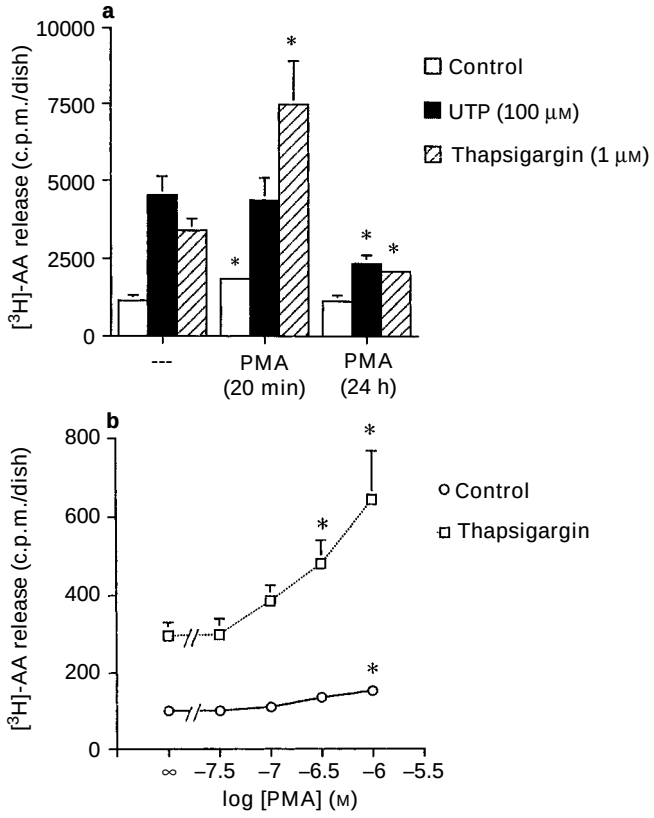

Figure 3 Effects of PMA on stimulus-induced AA release. (a) Cells were pretreated with media only or PMA (1 μM) for either 20 min or 24 h, after which basal, UTP (100 μM)- or thapsigargin (1 μM)-induced AA release was measured. (b) Cells were pretreated with media only or with PMA at the concentrations indicated for 20 min, then basal or thapsigargin (1 μM)-induced AA release was measured. Results are expressed as the mean of three independent experiments; vertical lines show s.e.mean. *Indicates $P<0.05$ as compared to the respective control response without PMA pretreatment.

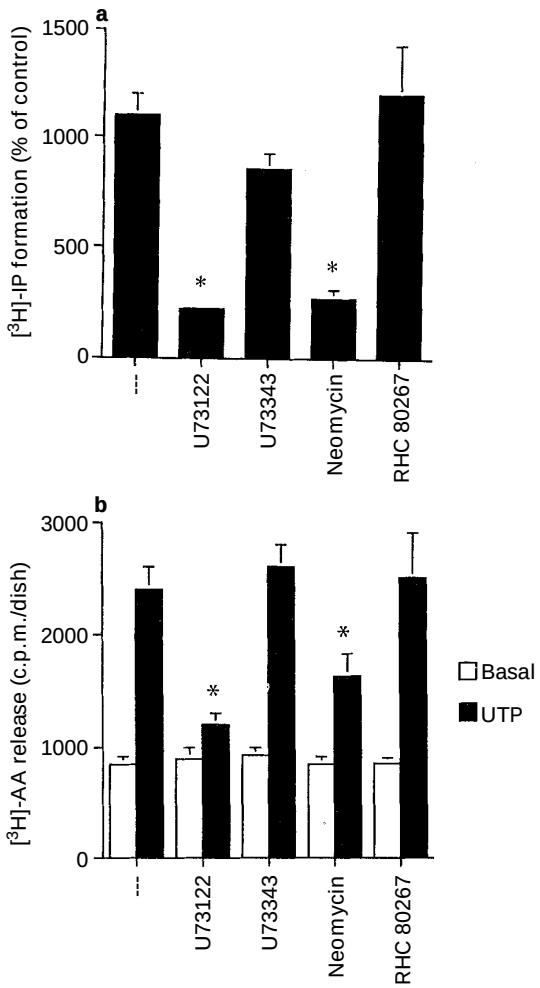

Figure 4 Effects of U73122, U73343, neomycin and RHC 80267 on UTP-induced IP formation and AA release. Cells were pretreated with U73122 (10 μM), neomycin (3 mM), U73343 (10 μM) or RHC 80267 (30 μM) for 20 min before stimulation of IP formation (a) or AA release (b) by UTP (100 μM). Results are expressed as the mean$\pm$s.e.mean of three independent experiments. *Indicates $P<0.05$ as compared to the UTP response without drug pretreatment.

butanol (which accelerates the production of phosphatidylbutanol from PA). We found that the net AA release caused by UTP was unaffected by the presence of either 300 μM propranolol (111$\pm$8% of control UTP response, $n=3$) or 1% butanol (103$\pm$7% of control UTP response, $n=3$).

Wortmannin, a known inhibitor of PI 4-kinase (Nakanishi *et al.*, 1995), can interfere with agonist-induced PI turnover via depletion of phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P_2), the substrate for PI-PLC. Wortmannin pretreatment attenuated the induction of AA in response to UTP in a concentration-dependent manner, but had no effect on thapsigargin-induced AA release, over the concentration range 10 nM–3 μM (Figure 5). Within this concentration range, wortmannin had no cytotoxic effect and failed to affect the basal level of AA release. When cells were treated with PTX (100 ng ml^{-1} for 24 h) and wortmannin (1 μM for 20 min) in combination, AA release in response to UTP was abolished (Figure 5b). Furthermore, the formation of IP in response to UTP was also inhibited in a concentration-dependent manner (Figure 6a,b). In order to investigate further whether wortmannin had a direct effect on cPLA$_2$, Ca^{2+}-stimulated AA release was measured in permeabilized cells. Figure 6c shows that wortmannin at concentrations up to 3 μM did not affect the activation of cPLA$_2$ in response to increasing Ca^{2+} levels.

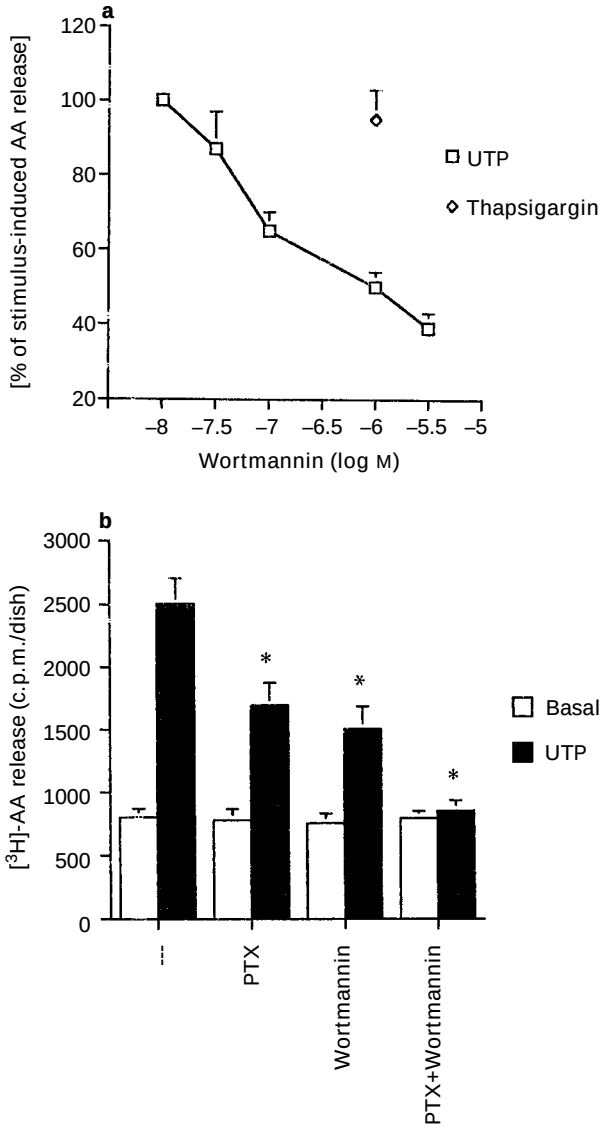

Figure 5 Concentration-dependent effects of wortmannin on stimulus-induced [^{3}H]-AA release. (a) Cells were pretreated with wortmannin at the concentrations indicated for 20 min, then UTP (100 μM)- or thapsigargin (1 μM)-induced [^{3}H]-AA release was measured. (b) Cells were pretreated with PTX (100 ng ml^{-1}) for 24 h, wortmannin (1 μM) for 20 min or both, before the addition of 10 μM UTP. Data represent the mean and vertical lines show s.e.mean, from three independent experiments. *Indicates $P<0.05$ as compared to the control response without wortmannin or PTX pretreatment.

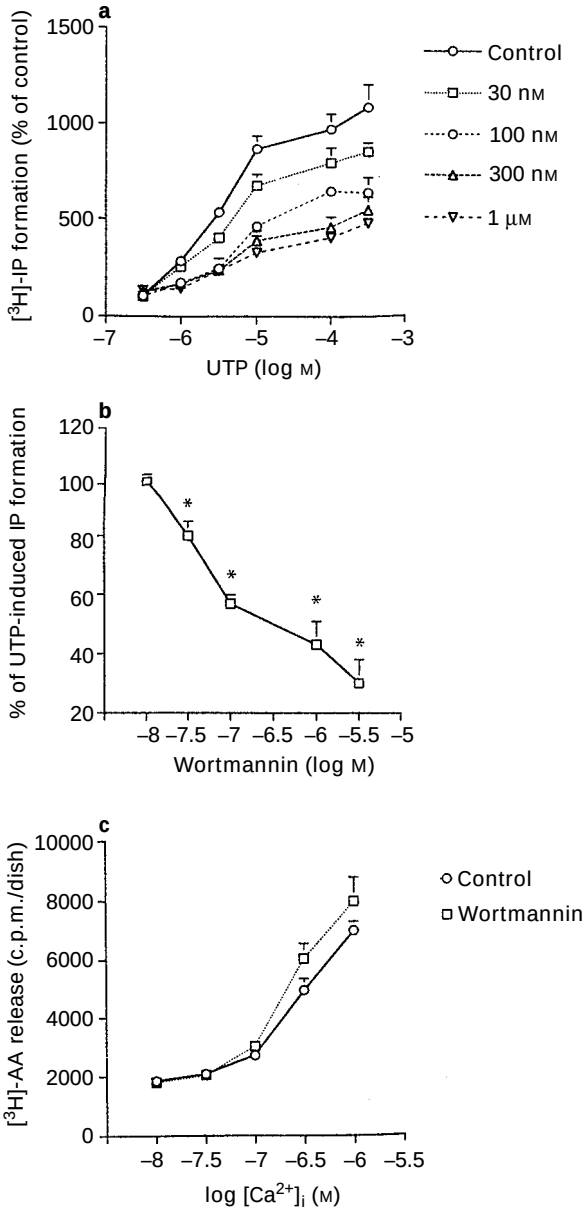

Figure 6 Effects of wortmannin on UTP-induced IP formation and Ca^{2+}-induced AA release. (a) Cells were pretreated with media only, or with 30 nM, 100 nM, 300 nM or 1 μM wortmannin for 20 min. Various concentrations of UTP were then added for 30 min, after which [^{3}H]-IP accumulation in the presence of 10 mM LiCl was measured. (b) Cells were pretreated with various concentrations of wortmannin for 20 min, after which UTP (10 μM)-induced [^{3}H]-IP accumulation was measured. (c) Cells labelled with [^{3}H]-AA overnight were permeabilized with 10 μM digitonin in a potassium glutamate buffer for 5 min, then washed and incubated in the potassium glutamate buffer containing various [Ca^{2+}] with or without 3 μM wortmannin. After 30 min incubation, [^{3}H]-AA released was determined as indicated in Methods. Results are expressed as the mean of three independent experiments; vertical lines show s.e.mean.

Correlation between AA release and $[Ca^{2+}]_i$ increase

As previously shown by Lin & Lee (1996), treatment of cells with UTP (100 μM) caused an increase in $[Ca^{2+}]_i$ from 183 ± 26 to 507 ± 40 nM ($n=13$) with a sustained plateau phase. Ca^{2+} mobilization displayed a similar dependence on UTP concentration to that previously shown for the activation of PI-PLC and PLA_2 (Lin & Lee, 1996). The net $[Ca^{2+}]_i$ increases were 133 ± 99 ($n=3$), 189 ± 86 ($n=4$), 251 ± 48 ($n=9$) and 324 ± 40 ($n=13$) nM in the presence of 0.1, 1, 10 and 100 μM UTP, respectively. To determine the effects of pharmacological manipulations on UTP-induced $[Ca^{2+}]_i$ response, the peak and sustained $[Ca^{2+}]_i$ increases caused by 100 μM UTP within 15 s and at 2 min, respectively, were quantified. In the absence of extracellular Ca^{2+} (following removal of $CaCl_2$ and addition of 1 mM EGTA), UTP (100 μM) induced only a transient $[Ca^{2+}]_i$ increase, with no plateau phase, indicating that the initial increase in $[Ca^{2+}]_i$ was probably due to the mobilization of intracellular Ca^{2+}, while the sustained increase was due to a Ca^{2+} influx. Likewise, thapsigargin (1 μM) stimulation caused a sustained increase in $[Ca^{2+}]_i$ from 169 ± 25 nM to 643 ± 73 nM ($n=5$) (Table 1), which also appeared to be dependent on extracellular Ca^{2+} influx.

As shown in Table 1, the $[Ca^{2+}]_i$ increases caused by UTP (100 μM) and thapsigargin (1 μM) were unaffected by pretreatment of cells with PTX (100 ng ml^{-1}, 24 h). However, short-term treatment (20 min) of cells with PMA (1 μM) resulted in an attenuation of the sustained increase in $[Ca^{2+}]_i$ induced by UTP, but did not affect its initial peak response. Long-term PMA pretreatment for 24 h inhibited the increase in $[Ca^{2+}]_i$ in response to UTP. The addition of 10 μM U73122 to RAW 264.7 cells did not affect the $[Ca^{2+}]_i$ response to thapsigargin, but it greatly diminished the UTP-stimulated sustained increase in $[Ca^{2+}]_i$. It was also found that SK&F 96365 (30 μM) did not alter the effect of UTP. By contrast, the response of cells to thapsigargin (1 μM) was unaffected by PMA pretreatment for 20 min or 24 h. The sustained but not the initial peak response of thapsigargin was found to be inhibited by SK&F 96365.

Discussion

To date, the mechanisms involved in the regulation of $cPLA_2$ are still not completely understood. The activation of $cPLA_2$ has been proposed to occur via a number of mechanisms, including those dependent on $[Ca^{2+}]_i$, protein phosphorylation and G proteins. Increased $[Ca^{2+}]_i$, which results in the translocation of $cPLA_2$ from the cytosol to the cell membrane (Clarke *et al*., 1991), seems to constitute an indispensable factor in the activation of $cPLA_2$ by agonists in a variety of cell types. The inhibitory effects of a nonselective PLA_2 inhibitor, BPB (Mayer & Marshall, 1993) and an irreversible $cPLA_2$ inhibitor, MAFP (Huang *et al*., 1996), indicate that both UTP- and thapsigargin-induced AA release by RAW 264.7 cells result from the activation of $cPLA_2$. The ineffectiveness of artistolochic acid (an inhibitor of $sPLA_2$, Vishwanath *et al*., 1988) ruled out the involvement of $sPLA_2$ in the UTP response. Results obtained with the fura-II method indicated that UTP induces a sustained increase in $[Ca^{2+}]_i$ in RAW 264.7 cells. Our experiments have underlined the critical role of agonist-induced influx of Ca^{2+} in the activation of $cPLA_2$. Firstly, a two fold increase in AA release induced by UTP in the presence of external Ca^{2+} was completely abolished by the removal of extracellular Ca^{2+} and substantially reduced by treatment with BAPTA/AM, an intracellular Ca^{2+} chelator. Secondly, thapsigargin, which increases $[Ca^{2+}]_i$ by distinct mechanisms, also evoked AA release. Thirdly, SK&F 96365, an inhibitor of capacitative Ca^{2+} entry (Merritt *et al*., 1990), was seen to have parallel inhibitory effects on thapsigargin-induced $[Ca^{2+}]_i$ elevation and AA release.

The coupling of UTP-induced AA release to PI-PLC activation was addressed by treating cells with three inhibitors of PI breakdown. U73122 and neomycin inhibit PI breakdown by interfering with the coupling of a G protein to PI-PLC (Smith *et al*., 1990) and binding of PtdIns (4,5)P_2 to PI-PLC (Slivka & Insel, 1988), respectively. Wortmannin is not only a potent inhibitor of PI 3-kinase and phospholipase D (PLD) (with IC_{50} of 3–5 nM and 57 nM, respectively) (Thompson *et al*., 1991; Ui *et al*., 1995), but also inhibits PI 4-kinase (with an IC_{50} of about 50 nM) (Nakanishi *et al*., 1995). The latter is a key enzyme in the formation of PtdIns (4,5)P_2, the substrate for PI-PLC. There is increasing evidence in support of the hypothesis that the supply of PtdIns (4,5)P_2 is a limiting factor in inositol phosphate production (Nakanishi *et al*., 1995). In accordance with this, we found that wortmannin, at a concentration between 0.1 and 3 μM, inhibited UTP-induced IP formation in a non-competitive manner and with no direct effect on the $cPLA_2$ activity triggered by Ca^{2+} in permeabilized RAW 264.7 cells. The inhibition of UTP-induced AA release by U73122, neomycin and wortmannin suggests that the activation of $cPLA_2$ by UTP is dependent on the PI-PLC signalling pathways. This suggestion is supported by the finding that the UTP-induced intracellular Ca^{2+} response was inhibited by U73122 and neomycin at the same concentrations that have been shown to inhibit the IP response. Thus we conclude that the inhibitory effects of U73122 and neomycin on AA release occur as a result of the inhibition of PI turnover, IP_3 formation and subsequent $[Ca^{2+}]_i$ rise.

The role of PKC in the stimulant effects of UTP and thapsigargin on AA release was investigated by examining the effects of PKC inhibitors and PMA-mediated PKC activation.

Table 1 Effects of pharmacological manipulations on UTP- and thapsigargin-induced $[Ca^{2+}]_i$ increase

	UTP		*Thapsigargin*	
Treatment	*Peak* (nM)	*Sustained* (nM)	*Peak* (nM)	*Sustained* (nM)
- - - - -	324 ± 40	265 ± 31 (13)	474 ± 43	217 ± 26 (5)
Ca-free	119 ± 23*	20 ± 13* (3)	133 ± 29*	12 ± 7* (3)
PTX (100 ng ml^{-1}, 24 h)	317 ± 32	250 ± 31 (3)	453 ± 30	237 ± 32 (3)
PMA (1 μM, 20 min)	309 ± 19	95 ± 18* (6)	437 ± 27	223 ± 15 (3)
PMA (1 μM, 24 h)	179 ± 29*	74 ± 18* (4)	459 ± 20	198 ± 18 (3)
U73122 (10 μM)	278 ± 21	93 ± 12* (4)	480 ± 22	200 ± 30 (4)
SK&F 96365 (30 μM)	310 ± 25	248 ± 16 (4)	455 ± 27	73 ± 11* (4)

The net $[Ca^{2+}]_i$ increases within 15 s (peak response) or at 2 min (sustained response) after UTP (100 μM) or thapsigargin (1 μM) addition were calculated. The values in the parentheses indicate the number of independent experiments.
*$P < 0.05$ as compared to the value without drug pretreatment.

Two lines of evidence support the theory that stimulation of AA release is dependent on PKC activation. Firstly, it has been shown that the down-regulation of PKC inhibits the effects of UTP and thapsigargin. Secondly, the PKC inhibitors, staurosporine, Ro 31-8220 (Davis *et al*., 1992) and Go 6976 (Martiny-Baron *et al*., 1993), also significantly attenuated AA release in response to UTP and thapsigargin. These results suggest the involvement of PKC in UTP-induced cPLA$_2$ activation. Although the *in vitro* phosphorylation of cPLA$_2$ from the murine macrophage cell line J774 has been demonstrated with exogenous PKC (Wijkander & Sundler, 1991), it was unclear whether this phosphorylation increased the catalytic activity of cPLA$_2$. Moreover, there is no evidence that PKC can directly phosphorylate cPLA$_2$ in intact cells. The present results seen in RAW 264.7 macrophages, where PMA slightly increased AA release when used alone and markedly potentiated the production of AA in response to thapsigargin, also support the conclusion that activation of PKC itself is an insufficient stimulus to increase AA release in RAW 264.7 cells. The slight inhibition of basal AA release by 10 μM Ro 31-8220 also suggests the involvement of endogenous PKC activity in spontaneous PLA$_2$ activity. Interestingly, we saw no potentiation of UTP-induced AA release by PMA in RAW 264.7 cells. This discrepancy can be explained by the distinct effects of PKC activation on the modulation of $[Ca^{2+}]_i$ in response to UTP and thapsigargin, i.e. although PKC activation of PMA did not alter the increase in $[Ca^{2+}]_i$ in response to thapsigargin, it did inhibit the upregulation of $[Ca^{2+}]_i$ by UTP. This is in agreement with our previous finding that PKC activation results in down-regulation of pyrimidinoceptor-mediated PI turnover (Lin & Lee, 1996). Thus, we suggest that PMA-induced potentiation of cPLA$_2$ activation can be counteracted by a simultaneous reduction in the UTP-induced rise in $[Ca^{2+}]_i$.

Recently, the phosphorylation of tyrosine residues on cellular proteins has been observed in response to stimuli which activate the AA cascade in cells including neutrophils (Gromez-Cambronero *et al*., 1991; Nahas *et al*., 1996), platelets (Nakashima *et al*., 1991), basophils (Yu *et al*., 1991; Connelly *et al*., 1991), Kupffer cells (Chao *et al*., 1992) and macrophages (Glaser *et al*., 1990; 1993). However, it is as yet unclear whether this represents an independent integral part of Ser/Thr protein kinase cascades. At present it seems unlikely that direct tyrosine phosphorylation of cPLA$_2$ is involved in the regulation of this enzyme since phosphorylation of cPLA$_2$ occurs exclusively on Ser residues (Lin *et al*., 1992; de Carvalho *et al*., 1996). Genistein, a tyrosine protein kinase inhibitor, has been shown to inhibit PAF-mediated PGE$_2$ production in P388D$_1$ macrophages (Glaser *et al*., 1990), and zymosan-, Ca^{2+} ionophore- and PMA-stimulated production of prostaglandin E$_2$ (PGE$_2$) and leukotriene C$_4$ (LTC$_4$) in murine peritoneal macrophages (Glaser *et al*., 1993). However, genistein (at concentrations up to 100 μM) had no effect on AA release by RAW 264.7 cells in response to UTP and thapsigargin. Thus, we suggest that unlike the action of lipopolysaccharide, the UTP- and thapsigargin-induced cPLA$_2$ activation in RAW 264.7 cells are independent of tyrosine kinase. Our previous study showed the sensitivity of lipopolysaccharide-induced AA priming action to 50 μM genistein (Lin, 1977). In accordance with our present results, it has been shown that genistein does not prevent the PMA- or zymosan-induced phosphorylation of cPLA$_2$, activation of MAP kinase or AA release in rat liver macrophages (Ambs *et al*., 1995).

There is accumulating evidence, from studies both *in vitro* and *in vivo*, that p42 MAP kinase is one of the kinases responsible for phosphorylating cPLA$_2$ and thus increasing cPLA$_2$ activity (Lin *et al*., 1993; Nemenoff *et al*., 1993; Qiu & Leslie, 1994; Ambs *et al*., 1995). Using PD 98059 an inhibitor of MEK (Dudley *et al*., 1995), we demonstrated that p42 MAPK may not be involved in UTP- and thapsigargin-induced cPLA$_2$ activation, although PD 98059 (30 μM) can inhibit PMA-mediated AA release and p42 MAPK activation in bovine pulmonary artery endothelial cells (our unpublished data).

PTX was found to inhibit the AA release caused by UTP and thapsigargin. The selective effect of PTX on UTP is unlikely to be due to the inhibition of agonist-induced PI turnover or to elevation of $[Ca^{2+}]_i$. In a previous study, we demonstrated that UTP-induced IP generation occurs via a PTX-insensitive Gq protein (Lin & Lee, 1996) and the results presented here show that PTX pretreatment has no effect on $[Ca^{2+}]_i$ elevation. To data, although the underlying mechanism for the regulatory roles of Gi/Go proteins on cPLA$_2$ activation is still unclear, several possibilities have been proposed. For example, Winitz *et al*. (1994) proposed an association between the Giα subunit and the activation of the Ras-Raf-MAPK cascade. Another possibility is that the $\beta\gamma$ subunits released from Gi/Go proteins can stimulate PLA$_2$ activity and AA release (Jelsema & Axelrod, 1987).

In addition to the pathway involving activation of PLA$_2$, AA can be generated via another PtdIns (4,5)P$_2$ metabolite, DAG, which is catalyzed by DAG lipase to liberate AA (Liscovitch, 1992). Using RHC 80267, a specific DAG lipase inhibitor, it has been shown that in liver macrophages, both DAG lipase and/or cPLA$_2$ pathways contribute to the release of AA in response to various stimuli (Dieter & Fitzke, 1993). The production of AA as a result of the hydrolysis of DAG is essential for glucose- and carbachol-induced insulin release (Konrad *et al*., 1994). The ineffectiveness of RHC 80267 in inhibiting UTP-induced AA release rules out the involvement of DAG lipase in AA generation by RAW 264.7 macrophages. Moreover, to explore the possible involvement of PA in potentiation of cPLA$_2$ activation, as previously described in rabbit platelets (Hashizume *et al*., 1994), we tested propranolol (an inhibitor of PA phosphohydrolase) and butanol (which accelerates the production of phosphatidylbutanol from PA). Neither of these inhibitors had any effect, thus ruling out the contribution of PA in UTP-induced AA release.

In conclusion, cPLA$_2$ activation caused by UTP and thapsigargin in RAW 264.7 macrophages primarily depends on the sustained elevation of $[Ca^{2+}]_i$ through different Ca^{2+} mobilization pathways. The activation of cPLA$_2$ due to PKC-dependent phosphorylation and a pertussis toxin-sensitive pathway downstream of the $[Ca^{2+}]_i$ increase is also involved.

This research was supported by a research grant from the National Science Council of Taiwan (NSC87-2314-B002-307).

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(Received June 9, 1997
Revised November 21, 1997
Accepted December 3, 1997)